Chapter 17

Allergen-Specific Immunotherapy Follow-Up by Measuring Allergen-Specific IgG as an Objective Parameter

Ernesto Akio Taketomi, Juliana Silva Miranda,
Jair Pereira da Cunha-Júnior and
Deise Aparecida de Oliveira Silva

Additional information is available at the end of the chapter

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Abstract

The clinical efficacy of the allergen-specific immunotherapy (AIT) has been well-documented using inhalant or hymenoptera-derived allergens in atopic patients with corresponding specific IgE antibodies. AIT is considered as the unique treatment that is capable of modifying the natural course of the allergic disease because it induces a variety of immunological mechanisms, with emphasis in the production of blocking IgG antibodies by IL-10-stimulated B cells due to the generation of Treg, Breg, or even Th2 cells. Thus, the measurement of specific IgG subclasses, particularly IgG4, to the crude extract or more importantly to allergen components, might be a useful and potential tool to follow-up objectively the patients undergoing AIT in addition to clinical parameters. In this chapter, the authors have emphasized a very sensitive and highly specific reverse ELISA, developed by them, to measure IgG subclasses directed to clinically relevant natural allergens that are undoubtedly better when compared to those obtained with recombinant counterparts. Such a technique may produce more authentic results taking into account the IgG subclass binding capacity to a particular allergen and might be a valuable and alternative method for monitoring activation of tolerance-inducing mechanisms in patients under AIT.

Keywords: allergen-specific immunotherapy, immunotherapy follow-up, blocking antibody, IgE, IgG4

1. Introduction

Allergen-specific immunotherapy (AIT) is indicated for atopic patients with IgE-mediated allergic diseases, particularly in allergic rhinitis, mild or moderate asthma and hymenoptera sting allergy. AIT is an effective treatment that aims to induce changes in immune response against specific allergen components derived from causal agents instead of the exteriorized symptoms, helping for modifying the natural course of the allergic disease and improving the patients' quality of life by the reduction of symptoms and medication use when naturally exposed to sensitized allergens. It involves a build-up phase that consists of the administration of gradually increasing levels of specific allergens until an effective dose that enables the reduction of the severity of the disease is reached, even in the presence of the natural allergen exposure [1].

On the one hand, the classical respiratory allergic disease is mediated by IgE antibodies to indoor or outdoor inhalant allergens through the development of Th2 cells that produce a well-known cytokine profile, including IL-4 and IL-13 [2]. These cytokines are crucial to cause antibody class switch on B cells to induce the synthesis of IgE antibodies, which in turn bind to mast cells and basophils that possess Fc epsilon receptor (FcεR types I or II) on their membranes, inducing the sensitization phase. In subsequent contacts, allergens containing genuine- or cross-reactive epitopes capable to cross-link to IgE bound to target cells can activate these cells, with consequent release of preformed and newly formed vasoactive mediators. The preformed mediators (histamine) are responsible for early phase symptoms and newly formed those (leukotrienes and cytokines) for inducing a late-phase response, characterizing the type I hypersensitivity reaction [3].

On the other hand, the administration of allergens by AIT has been proved to cause early allergen-specific mast cell desensitization, likely as a consequence of the development of regulatory T cells (Tr1 cells) that particularly produce IL-10, which induces antibody class switch on B cells to produce IgG4 antibody subclass. An alternative way to produce other subclasses of IgG can be achieved due to the fact that AIT can provoke immune deviation from Th2 in favor of Th1 responses that culminate in the production of IFN-γ, which induces B cells to produce IgG1 subclass [4]. In the initial phase of AIT the immunological response involves the production of IgG1 antibodies whereas IgG4 is the dominant subclass in prolonged AIT. Therefore, IgG antibodies induced by AIT may act as blocking antibodies, reflecting in the reduction of mast cell activation and degranulation as well as competing with IgE antibodies for allergen binding, blocking IgE-dependent mast cell activation and inhibiting IgE-facilitated allergen presentation [1].

Currently, there is no routine laboratorial test for the detection of allergen-specific IgG antibodies, particularly IgG1 and IgG4 subclasses. Physicians, who assist patients with respiratory allergy that have been submitted to AIT, are following the treatment of such patients only by subjective clinical parameters. The possibility of following such patients under AIT by laboratorial evaluation of allergen-specific IgG1 and/or IgG4 levels has stimulated researchers to develop objective methods for quantifying allergen-specific IgG antibodies.

The detection of IgG antibodies, particularly IgG1 and IgG4 subclasses, against specific allergenic components, such as the major allergens of *Dermatophagoides pteronyssinus* (Der p 1 and

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Der p 2) would indicate the development of a physiological response, i.e., a defense response against dust mite allergens [5]. Production of specific IgG4 antibodies to relevant allergenic components has been associated with the protective activity due to its function as blocking antibody through mechanisms of competition for allergen between IgG4 and cell-bound IgE antibodies [6]. Thus, the role of specific serum IgG subclasses, particularly IgG4, might be considered as a good marker of protective or blocking antibody that may be useful for monitoring activation of tolerance-inducing mechanisms in patients under AIT.

Therefore, it becomes particularly interesting the development of a method for quantifying IgG subclasses against clinically relevant allergens. These antibodies can be detected in the serum or other biological fluids, such as saliva from patients with allergic respiratory disease using an immunoenzymatic technique (reverse ELISA) and allergen component-specific monoclonal antibodies for monitoring patients under AIT. This assay represents a potential tool for monitoring patients with respiratory allergy, especially during AIT.

2. Allergic response

2.1. Sensitization phase

The balance of the different subsets of T helper cells such as Th1, Th2 and Treg with their cytokine profiles supports the maintenance of the homeostasis of the immune system. The breakdown of this balance among Th1, Th2 and Treg cells leads to excessive activation of Th1 or Th2 cells, culminating in the development of autoimmune diseases or induction of IgE-mediated allergic diseases, respectively [2]. Allergies are one of the most prevalent diseases in the world, once they are a result of a breakdown in the immune tolerance that individuals usually have to food, inhalant and insect venom allergens [7, 8]. These diseases have a mechanism of response based on an interaction of the innate and adaptive immune system, with interaction of various cell types, cytokines, chemokines and costimulatory signals responsible for different T-cell responses [9].

Th2-cell subset is induced in a classical respiratory allergic disease, triggering a pathogenesis related to several indoor or outdoor inhalant allergens as excretions of house dust mite and cockroaches, animal dander, pollens and fungal spores, among others [10]. In addition, the dose and function of the allergen are relevant for allergic sensitization [7]. This step is the first event of the classical pathogenesis, which is mediated by producing specific IgE antibodies directed to epitopes derived from inhalant allergens through the development of Th2 cells. First of all, the allergens can pass through the epithelial tissue cells of the respiratory tract or directly bind in receptors of innate immune cells. Then, allergens are uptaken and processed by professional antigen-presenting cells (APCs), as dendritic cells (DCs), that present peptides through class II major histocompatibility complexes (MHC II) to naive CD4+ T cells located in the submucosal layer, driving to effector and memory T cells of the Th2 phenotype (**Figure 1**) [11]. For that, APCs mediate the production and secretion of crucial cytokines as IL-4, characterizing the occurrence of the third signal of the immune response, which will be responsible for the STAT-6 activation and subsequently GATA-3 (GATA-binding protein 3

transcription factor) upregulation [12]. Besides the antigenic peptide presentation (first signal of the immune response), the participation of costimulatory molecules (second signal) is necessary to reach the development of Th2 cells by increasing the expression of genes encoded on 5q31-33 chromosome (**Figure 1**). These genes are associated to IL-3, IL-4, IL-5, IL-9, IL-13 cytokines and granulocyte-macrophage colony stimulating factor (GM-CSF) codification, related to Th2 pathway [13]. Some of these cytokines, such as IL-4 and IL-13, are responsible for switching the antibody class on B cell to induce the synthesis of IgE antibodies, which bind certain target cells that possess Fc epsilon receptor (FcεR) type I (high-affinity) or type II (low-affinity) on their membranes like mast cells and basophils, leading to the establishment of the sensitization phase [14].

Some allergens, as proteolytic protein or lipopolysaccharide (LPS), can stimulate other bias of Th2 response, once the linkage of proteolytic allergens to pattern recognition receptors (PRRs) like protease-activator receptors (PARs), or a linkage of LPS to toll-like receptors (TLRs), both localized in barrier epithelial cells, or even the production of reactive oxygen species (ROS) by damaged cells can promote various effects that drive to a proinflammatory response. For instance, PARs and TLRs can be a trigger to epithelial cells to produce cytokines, like thymic stromal lymphopoietin (TSLP), interleukin-25 (IL-25) and IL-33 related to allergic inflammation (**Figure 1**) [15, 16]. IL-25 and IL-33 can upregulate NFκ-B, together with TSLP that activate

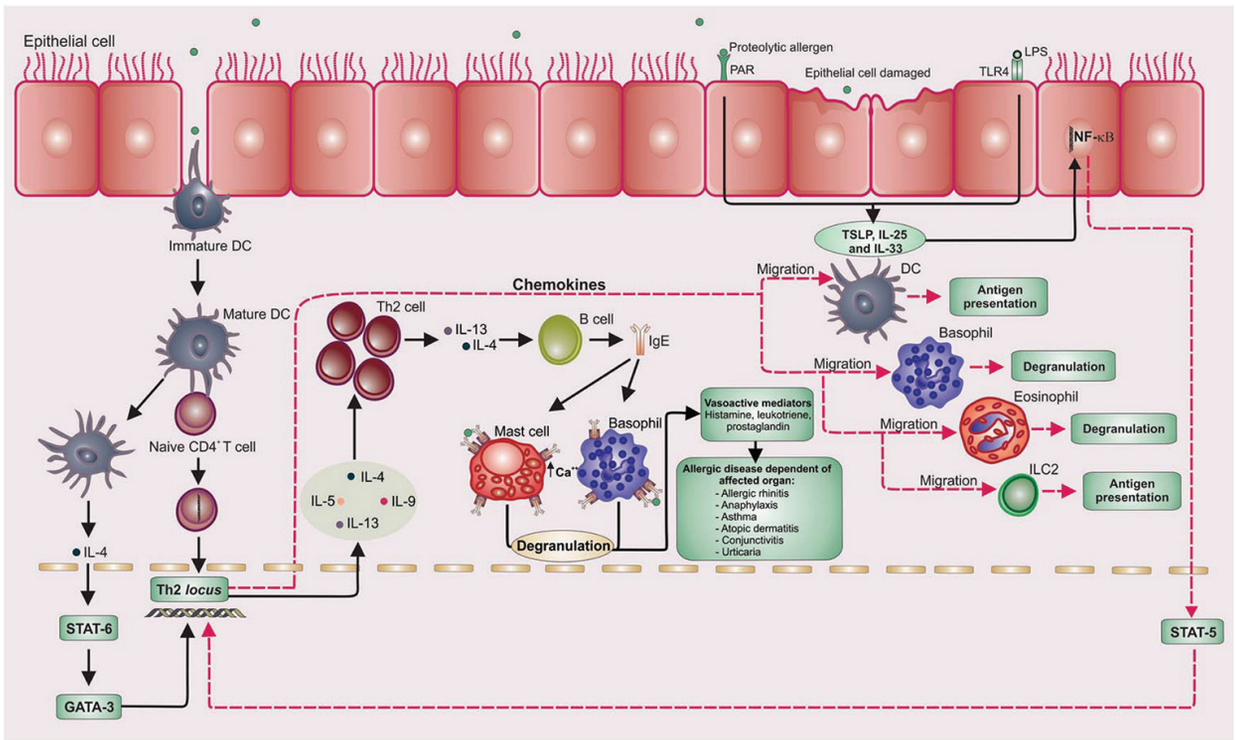

Figure 1. Innate and adaptive allergic immune response. Sequential events for allergen sensitization and triggering the immune response that generates different allergic diseases depending on the affected organs are shown. $CD4^+$: cluster of differentiation 4; DC: dendritic cell; GATA-3: GATA-binding protein 3 transcription factor; ILC2: group 2 innate lymphoid cell; LPS: lipopolysaccharide; NF-κB: nuclear factor kappa B; PAR: protease-activated receptor; STAT-5: signal transducer and activator of transcription 5; STAT-6: signal transducer and activator of transcription 6; TLR4: Toll-like receptor type 4; TSLP: thymic stromal lymphopoietin.

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STAT-5 promoting an increase of Th2 genes regulation. This way stimulates the production of chemokines and cytokine release that contribute to cell migration, especially DCs, basophils and eosinophils as well as group 2 innate lymphoid cells (ILC2) involved in allergic responses (**Figure 1**) [17, 18].

Taken together, there are mechanisms that promote a Th2 pathway by GATA-3 upregulation induced especially by IL-4-activated STAT-6, or a Th2 route in which GATA-3 expression is induced in an IL-4 and STAT-6-independent manner [2]. Thus, the maintenance of Th2 responses by environmental allergens is related to the type of recognition of the allergens in the epithelial barrier, which promotes the linkage of innate and adaptive responses [19].

2.2. Effector phase

Allergic subjects besides mast cells and eosinophils with a greater number of IgE receptors, have an increase of IgE-producing B cells stimulated by IL-4 and IL-13-secreting Th2 subset [20]. In a subsequent contact with allergens that contain genuine- or cross-reactive epitopes capable to cross-link IgE bound to target cells, calcium-dependent activation of these cells can occur with release of preformed vasoactive mediators as histamine responsible for the early phase symptoms and newly formed vasoactive mediators like leukotrienes and cytokines for late phase symptoms (**Figure 1**) [21]. These mediators are characterized by the maintenance of long-lasting symptoms due to the continued tissue inflammation and injury, characterizing typically the type I hypersensitivity reaction. Therefore, maturation of eosinophils induced especially by IL-5 and basophils by IL-3 and IL-4 are the main secreting effector cells of inflammatory mediators observed in the classical allergic response [12]. Local symptoms or systemic anaphylaxis may be observed depending the affected organ and tissues in a particular individual response to sensitized allergens (**Figure 1**) [21].

The intensity of the immune response to allergens is crucial to develop an allergic condition mediated by IgE antibody, or a healthy condition depending on the individual gene susceptibility, environmental pollutants, features of allergens, among others [22–24]. Other antibody classes have been analyzed because of this variation of response between allergic and healthy subjects, such as IgA and IgG subclasses [23–25]. In healthy individuals, B cell response to house dust mite allergens ranges from no response to predominantly production of IgG antibodies specific to allergens, particularly IgG1 or IgG4 subclass, in the absence or low concentration of IgE. Differently, IgG levels, particularly IgG4 subclass, have also been detected in allergic subjects in addition to high levels of IgE, but IgG1 levels have been found at similar levels in both healthy and allergic individuals [26, 27].

3. Cellular and molecular mechanisms of AIT

Allergen-specific immunotherapy (AIT) is performed by the administration of increasing concentrations of allergens (build-up phase) up to maintenance doses, mainly given by subcutaneous, epicutaneous, oral, sublingual, or recently by intralymphatic route. AIT aims to induce

changes on the immune response of allergic individuals, drawing a state of allergen-specific tolerance, which contributes with a curative effect for a long period of time [28–32].

The cellular and molecular mechanisms of AIT are diverse, involving the very early mast cell and basophil desensitization, effect on antigen-presenting cells, modulation of T and B cell repertories as well as modification of allergen-specific antibody responses (**Figure 2**) [32].

Although AIT reduces the allergic inflammation mediated by IgE-dependent mechanism over the time, a very early effect on basophil and mast cell activation status is observed just after the initiation of the therapeutic regimen, leading to a lower risk to develop anaphylactic manifestations [33–35]. The subjacent mechanism of basophil and mast cell desensitization has not been elucidated yet; however, some clues highlight this issue. AIT leads to a controlled releasing of histamine and leukotrienes by basophil and mast cells after allergen administration, producing a gradual reduction of granule content of the inflammatory mediators in these cells in patients submitted to immunotherapy [31, 33, 36], although there is not a direct evidence of diminution of intracellular vasoactive mediator amount by histological analysis. Short-term venom immunotherapy induces desensitization of FcεRI-mediated basophil response. The levels of mRNA and FcεRI cell-surface expression decreased in basophil cells from patients submitted to venom immunotherapy, indicating that the reduction in FcεRI

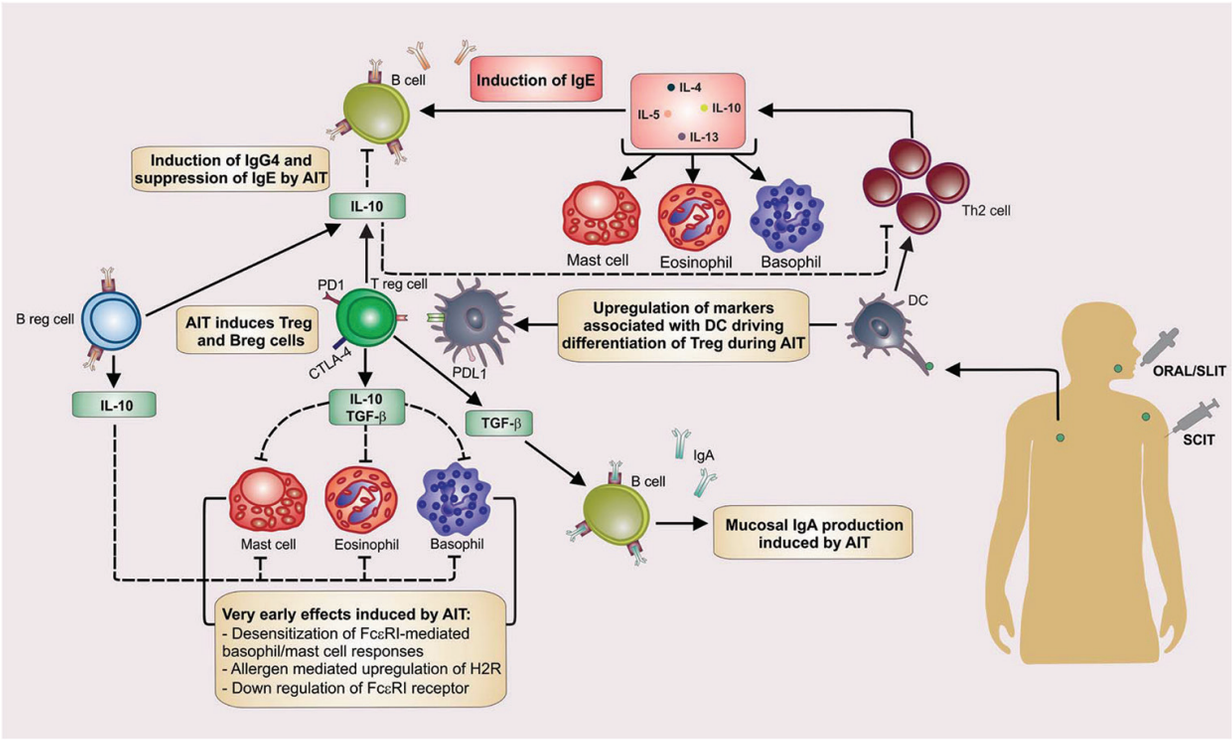

Figure 2. Immunological changes induced by allergen-specific immunotherapy (AIT). Desensitization of basophil/mast cells and upregulation of markers associated with dendritic cells (DCs) driving differentiation of IL-10-producing Treg and Breg cells and subsequent activation of B cells to synthesize allergen-blocking factors, particularly IgG4 and suppression of IgE antibodies during AIT are shown. AIT: allergen-specific immunotherapy; Breg cell: regulatory B cell; CTLA-4: cytotoxic T lymphocyte antigen-4; DC: dendritic cell; FcεRI: high-affinity receptor for the Fc region of immunoglobulin E (IgE); H2R: histamine H2 receptor; PD1: programmed death-1 receptor; PDL1: programmed death ligand-1; SCIT: subcutaneous immunotherapy; ORAL/SLIT: oral/sublingual immunotherapy; Treg cell: regulatory T cell.

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expression contributes to the phenomenon of the early basophil desensitization observed after AIT [37, 38]. On the other hand, AIT also provokes an allergen-mediated upregulation of the type 2 histamine receptor (H2R) gene, which was associated with the suppression of FcεRI-mediated basophil activation, inducing a tolerogenic response (**Figure 2**) [34, 39]. The engagement of H2R with its agonists prevents further histamine and leukotriene releasing as well as IL-4 and IL-8 production by basophil cells [34]. The molecular mechanism involved in H2R-dependent basophil desensitization is supposed to be mediated by the cAMP pathway because the stimulation with H2R agonist or with a direct cAMP inducer was able to inhibit the FcεRI-mediated basophil activation [34]. In this way, the increase of concentration of cAMP activates PKA (Protein Kinase A, the principal intracellular target of cAMP), which in turn decreases the intracellular calcium influx, thus preventing FcεRI-dependent basophil and mast cell degranulation [22].

Antigen-presenting cells, particularly dendritic cells (DC), display an important role in the induction of allergic diseases driving Th2 responses and the IgE-dependent pathophysiologic mechanism. Some evidences reveal that AIT can affect directly the phenotype of the antigen-presenting cells correlating with clinical improvement in patients with allergic diseases [40–42]. A regulatory dendritic cell signature correlating with the clinical efficacy after allergen-specific sublingual immunotherapy (SLIT) has been observed in peripheral blood mononuclear cells (PBMCs) from clinical allergic responders in comparison with nonresponders or patients that received only placebo [40]. Likewise, a report using transcriptomic and proteomic approaches demonstrated that PBMCs from allergic patients downregulate the expression of markers related with DC driving the differentiation of Th2 cells, whereas upregulate markers associated with DC driving differentiation of T regulatory cells, after only 4 months of SLIT. These results indicate that AIT has an early effect on antigen-presenting cells that trigger the Th2 downregulation [42]. Therefore, the changes evoked during AIT regimen on antigen-presenting cells, with a predominance of DC tolerogenic subsets inducing the development of T regulatory (Treg) cells, may be part of the mechanism behind of the therapeutic efficacy observed in AIT (**Figure 2**).

The induction of the allergen-specific tolerance is a pivotal event required in AIT procedures by generating allergen-specific Treg cells [43], responsible for maintaining immune homeostasis. Treg cells have been characterized by stable expression of CD25, CD4 and FOXP3 (Forkhead box protein 3), expression of suppressive surface molecules, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programed death 1 (PD1) and secretion of IL-10 and TGF-β cytokines [31]. Accordingly, increased numbers of Treg cells were also detected in nasal mucosa correlating with clinical efficacy after AIT, supporting the importance of these cells on tolerogenic phenomenon observed in patients upon AIT [44].

TGF-β produced by Treg cells is a potent inhibitor of Th2, Th1 and Th17 effector response and has been associated with the suppression of seasonal allergic inflammation [31] and production of mucosal allergen-specific IgA after AIT [30, 45, 46]. Likewise, IL-10 produced by Tr1, Treg and Breg cells were markedly increased after AIT in allergic individuals and those cells were also associated with suppressor effect observed in several immunotherapeutical protocols [47–49].

IL-10 acts as a potent suppressor cytokine, reducing the production of proinflammatory cytokines by mast cells, decreasing the eosinophil functions and also downregulating the expression of MHC class II and costimulatory molecules on surface of monocytes/macrophages and DCs, thus preventing allergen-induced Th2 activation [50–53]. Importantly, IL-10 is related with the antibody class switch on B cells, favoring the production of IgG4 subclass, a dominant antibody subclass in late phase response of AIT, which is associated with a gradual decreasing of IgE levels. Alternatively, AIT can also provoke immune deviation from Th2 in favor of Th1 responses that culminate in the production of IFN-γ, inducing preferentially B cells to produce IgG1 subclass directed to allergenic components present in the formulation of the AIT [31].

Therefore, allergen-specific IgG antibodies induced during AIT may act as blocking antibodies, reflecting in the reduction of mast cell activation and degranulation due to its competition with IgE antibodies for allergen binding and inhibiting IgE-facilitated allergen presentation [54].

4. Immunotherapy follow-up

In addition to clinical parameters like improvement in symptoms and medication scores that are subjective, it should be very helpful if the medical assistant had also objective parameters such as complementary laboratorial tests for the follow-up of allergic patients under AIT.

Considering the several cellular and molecular mechanisms involved in AIT described before, such as the determination of the type 2 histamine receptor (H2R), basophil activation test, or a procedure for measuring regulatory dendritic cell signature, all of which can be correlated with the clinical efficacy of AIT, it becomes evident that complex methods should be employed and certainly would be difficult to be applied in the routine analysis.

Therefore, we can accomplish that measurement of specific IgG, particularly of the IgG4 subclass, might be used for monitoring patients receiving AIT, since it will be more simple and feasible in any clinical analysis laboratory. In this context, a previous study has demonstrated a lack of correlation between venom-specific total IgG levels and prediction of systemic reactions, concluding that measuring specific IgG antibodies is not useful for monitoring AIT. In this study, the authors postulated that IgG subclasses could be probably involved, since the clinical improvement is not necessarily reflected in the total IgG antibody titre [55]. However, other investigators have found a correlation between low levels of venom-specific IgG and a greater risk of anaphylaxis in patients submitted to venom allergen immunotherapy during 4 years and the opposite was also true, a lower risk of systemic reaction could be observed in those patients with high levels of venom-specific IgG, concluding that the measurement of specific IgG is useful and beneficial, especially for advising greater risk of anaphylaxis in patients who present low levels of specific IgG [56]. An interpretation that we can point out is the existence of two groups of patients who are receiving AIT; one group includes the good responders and another those patients that are non- or low-responders and such fact can be associated to their intrinsic genetic features, particularly related to the specific type of HLA (human leukocyte antigen). Also, we need to consider the presence of pre-existing levels of allergen-specific IgG subclasses before AIT, since the patients themselves may present

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stimulation of their immune system for attempting to synthesize blocking antibodies as an autoregulatory mechanism.

Recent experimental study using a high-dose cutaneous exposure to *Dermatophagoides pteronyssinus* mite extract has shown to induce effective blocking IgG production, supporting that the detection of increased IgG antibody titres is a promisor marker of clinical efficacy of AIT [57].

In addition, a study employing a nonclassical allergen intralymphatic immunotherapy using a modular antigen transporter Fel d 1 (MAT-Fel d 1) has found a strong increase in allergen-specific IgG4 levels and some increase in IgG2 antibody subclasses, but this procedure was not able to stimulate the production of IgG1 and IgG3 subclasses [58].

The production of specific IgG antibodies to allergens, especially IgG4 subclass, is the most important immunological change induced by AIT [59–62]. However, in some studies there is a lack of correlation between increased IgG4 titres and clinical improvement [55], since the induction of IgG4 blocking antibodies may not be reflected in serum or other biological fluid samples, requiring bioassays as the inhibition of IgE-facilitated allergen presentation for its possible detection [63]. Accordingly, production of specific IgG4 antibodies to relevant allergenic components has been associated with the protective activity due to its function as blocking antibody through mechanisms of competition for allergen between IgG4 and cell-bound IgE antibodies [6]. In this context, several other investigators have found that clinical improvement after mite AIT was associated with increased levels of serum specific IgG4 or ratio of specific IgG4/IgG1 [64–66].

Unfortunately, there is no current routine laboratorial test for the detection of allergen-specific IgG antibodies, particularly IgG1 and IgG4 subclasses, against crude allergen extract and/or clinically relevant allergen components that could be used as a useful tool for monitoring AIT. Physicians, who assist patients with respiratory allergy that have been submitted to AIT, are following the treatment of such patients only by clinical parameters (symptoms and medication scores) that are very subjective. The possibility of following such patients under AIT using allergen-specific IgG1 and/or IgG4 antibody measurements will enable to monitor those patients by using objective parameters in association with subjective clinical parameters. This fact has stimulated researchers to develop objective methods for quantifying those allergen-specific IgG antibodies.

In 2001, our group has developed a reverse ELISA technique for quantifying Der p 2 allergen-specific IgE antibodies, using capture Der p 2-specific monoclonal antibodies. This technique was developed with the intention of helping the allergy diagnosis by means of a molecular allergen component, since the presence of Der p 2 allergen-specific IgE antibodies indicates the occurrence of an allergic response in the patient. It has also been demonstrated that this technique has a higher sensitivity related to conventional ELISA [67].

On the other hand, the detection of specific IgG antibodies or particularly IgG1 and IgG4 subclasses, against Der p 2, or against any other specific allergenic component, would indicate the development of a physiological response, i.e., a defense response against dust mite allergens.

Thus, on the basis of the information described above, it becomes particularly interesting to develop a method for quantifying IgG antibody subclasses against clinically relevant allergens. These antibodies can be detected in the serum or other biological fluids, such as saliva from patients with allergic respiratory disease using an immunoenzymatic technique (reverse ELISA) and relevant monoclonal antibodies for monitoring patients under AIT.

5. Method for measuring allergen-specific IgG subclasses

Part of our group has developed a reverse ELISA technique as described in the European patent application registered as EP 2232265, providing a method for measuring allergen-specific IgG antibody subclasses, including IgG1, IgG2, IgG3 and IgG4, for monitoring patients with allergic diseases under AIT [68]. As illustrated in **Figure 3**, allergen-specific monoclonal antibodies, for example, anti-Der p 1 or anti-Der p 2, are bound to ELISA microtitration plates in order to capture the corresponding natural allergens, Der p 1 or Der p 2, respectively, present in the crude *D. pteronyssinus* extract, which subsequently interacts with specific IgG antibodies existent in serum samples or other biological fluids from allergic patients. Those antibodies are later detected by the addition of mouse monoclonal antibodies, against human IgG subclasses, preferentially IgG4, labeled with biotin and, subsequently are incubated with the streptavidin-peroxidase enzymatic conjugate. Reaction is revealed by the addition of the enzymatic substrate (hydrogen peroxide) diluted in a chromogenic buffer [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) – ABTS] and absorbance is determined in a microtitration plate reader, at 405 nm.

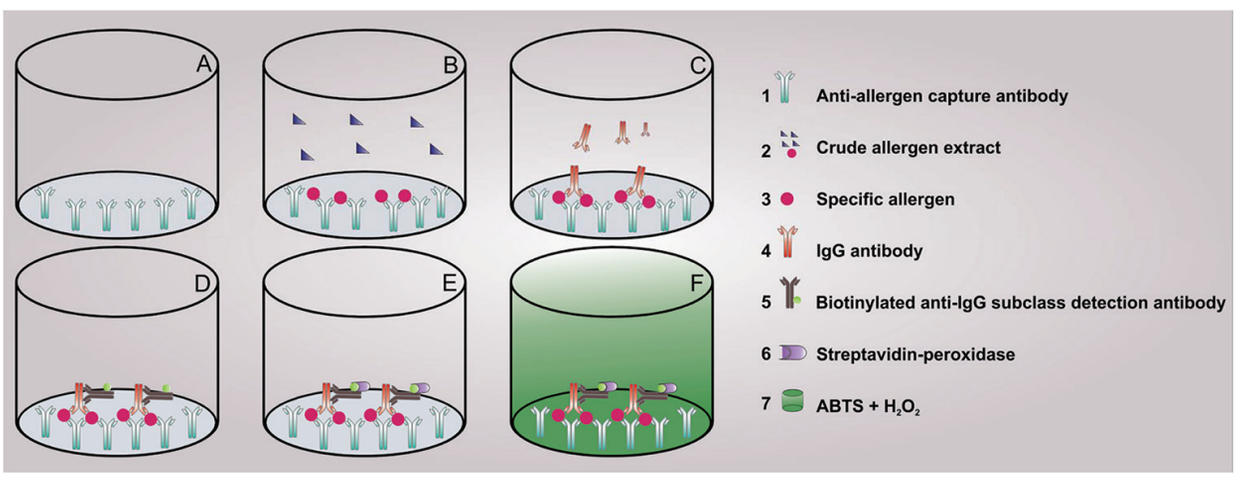

Figure 3. Representative diagram (sequential steps A to F) of the reverse enzyme-linked immunosorbent assay (ELISA). (1) Capture allergen-specific (Der p 1 or Der p 2) monoclonal antibody; (2) crude *Dermatophagoides pteronyssinus* extract; (3) allergen (Der p 1 or Der p 2) present in crude extract; (4) allergen-specific IgG antibody present in serum samples or other biological fluids from allergic patients; (5) monoclonal antibody against human IgG subclass (preferentially IgG4) labeled with biotin; (6) streptavidin-peroxidase enzymatic conjugate; (7) reaction is revealed by the addition of enzymatic substrate (hydrogen peroxide) diluted in a chromogenic buffer (ABTS) and absorbance is determined in a plate reader at 405 nm (Taketomi EA and Silva DAO, 2016, found in European Patent Office EP 2232265 [68]).

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The reverse ELISA (rELISA) technique for the detection of IgG antibody subclasses has a great advantage over others that use indirect ELISA [69, 70]. It does not require purified allergens or antigens, which are often too expensive or difficult to obtain in a purified and isolated form, since the natural allergen components present in the crude allergen extract are bound on the microtitration plate by the capture allergen-specific monoclonal antibody. Another advantage of this assay is that it does not require specific and exclusive equipment, avoiding a direct dependence between the producers of the diagnostic kits or the diagnostic equipment and the consumers.

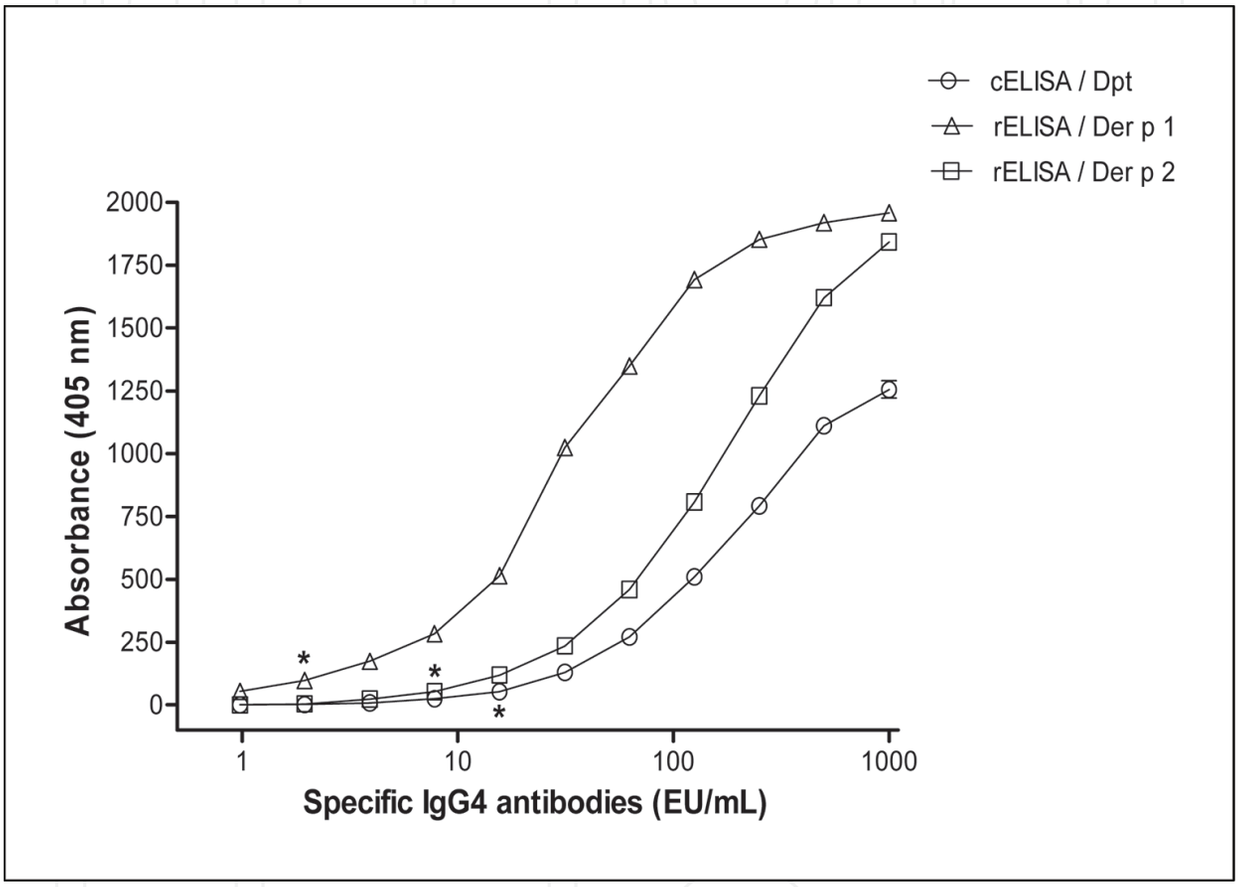

Figure 4. Sensitivity of cELISA and rELISA for the measurement of IgG4 antibodies to the crude extract of *Dermatophagoides pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2). The sensitivity of each assay is indicated by the asterisk (Taketomi EA and Silva DAO, 2016, found in European Patent Office EP 2232265 [68]).

The rELISA assay also demonstrated higher sensitivity than the conventional ELISA (cELISA) in the measurement of allergen-specific IgG subclasses, particularly IgG4 antibodies, to the crude *D. pteronyssinus* (Dpt) extract and its major allergens (Der p 1 and Der p 2), using a pool of reference sera obtained from mite-allergic patients (**Figure 4**). The sensitivity of each assay was 15.6 EU/mL for cELISA-Dpt, 1.9 EU/mL for rELISA-Der p 1 and 7.8 EU/mL for rELISA-Der p 2. Likewise, specificity of rELISA for the measurement of allergen-specific IgG subclasses, particularly IgG4 antibodies to the major allergens (Der p 1 and Der p 2) was shown

to be higher than cELISA for the detection of IgG4 to crude Dpt extract as determined by inhibition assays (**Figure 5**). All assays showed a dose-dependent manner inhibition when a pool of reference sera containing allergen-specific IgG4 antibodies was incubated with increasing concentrations (0.15–15,000 AU/mL) of the crude Dpt extract as inhibitor antigen. Inhibition was higher than 80% for all assays, with 88% for cELISA-Dpt, 82% for rELISA-Der p 1 and 89% for rELISA-Der p 2, when the highest concentration of Dpt allergen extract was used.

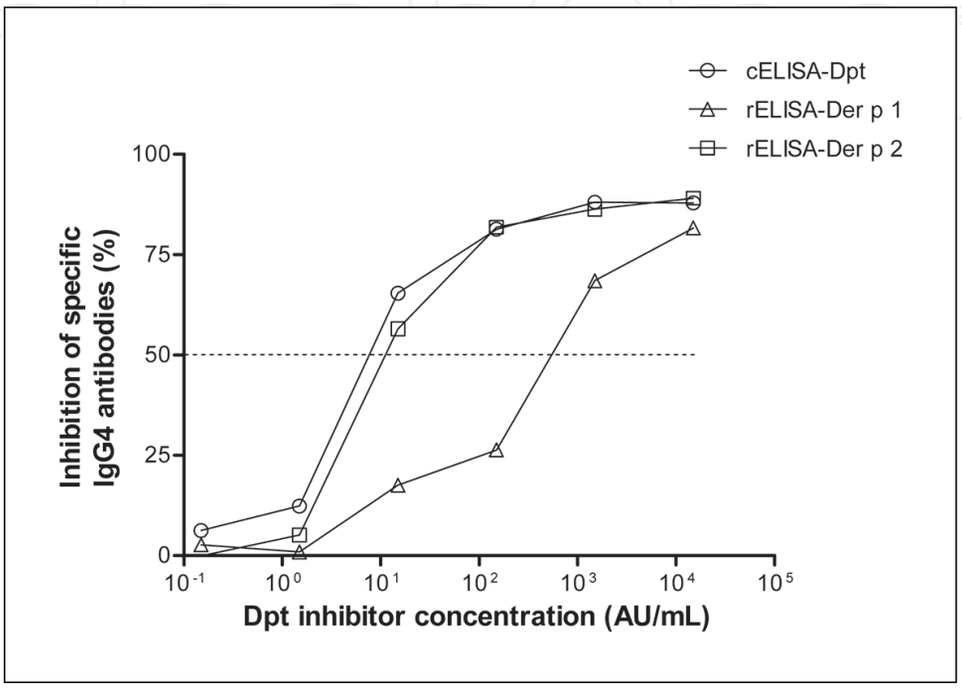

Figure 5. cELISA and rELISA specificity for IgG4 antibodies to the crude extract of *Dermatophagoides pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2) using competitive inhibition assays. Mite-allergic patient sera were preadsorbed with different concentrations of Dpt inhibitor antigen and then assayed in each cELISA and rELISA for measurement of specific IgG4 antibodies. Data represent the percentage of inhibition in each assay (Taketomi EA and Silva DAO, 2016, found in European Patent Office EP 2232265 [68]).

In our previous study [71], rELISA was also employed for monitoring specific IgG4 levels to *D. pteronyssinus* major allergens (Der p 1 and Der p 2) along with cELISA for measuring IgG4 levels to crude Dpt extract in serum samples of two groups of mite-allergic patients under AIT by subcutaneous route: one active DPT group, receiving the *D. pteronyssinus* extract and another placebo group. Serum samples were analyzed in two time-points, day 0 and after 1 year of treatment. As shown in **Figure 6**, patients of the active group (DPT) had increased levels of IgG4 to *D. pteronyssinus* extract and its major allergens, particularly to the Der p 1 allergen component, after 1 year of therapy as compared to patients without active immunotherapy (placebo group). Also, there was a significant increase of serum IgG1 levels to *D. pteronyssinus* extract and Der p 1 allergen component in patients that had received active immunotherapy in contrast with those patients belonging to the placebo group [71].

Furthermore, we were also able to show a significant increase in IgG1 and IgG4 levels to *D. pteronyssinus*, Der p 1 or Der p 2 allergen components after 12 and 18 months of sublingual immunotherapy using *D. pteronyssinus* extract. In contrast, patients receiving placebo did not

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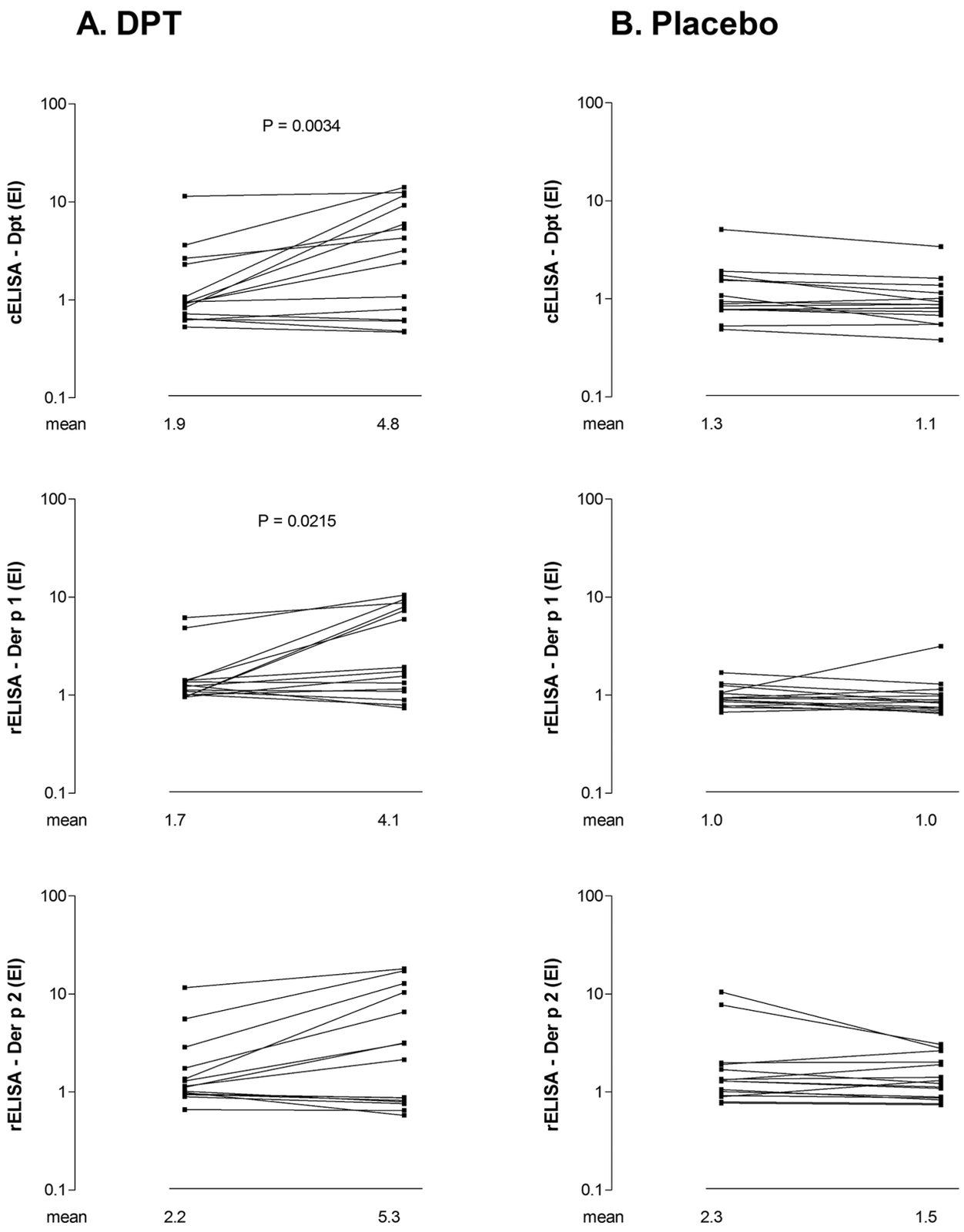

Figure 6. Levels of IgG4 antibodies to the crude extract of *Dermatophagoides pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2) determined by cELISA and rELISA in sera from patients randomized to two treatment groups: (A**)** active DPT (Dpt extract; *n* = 15) and (B**)** Placebo (*n* = 15). Antibody levels are expressed in ELISA indices (EI) as individual values on day 0 and after 1 year of treatment and connected with a line; the mean EI values for each of those two time-points are also indicated. Significant differences before and after treatment within the groups were determined by the Wilcoxon signed-rank test (Taketomi EA and Silva DAO, 2016, found in European Patent Office EP 2232265 [68]).

show any increases in IgG1 or IgG4 antibody levels to crude *D. pteronyssinus* extract or its major allergen components in that studied period of time [30].

Thus, our studies have shown that increased levels of allergen-specific IgG subclasses, particularly IgG4 and IgG1, can be detected after variable period of AIT in the serum of patients receiving mite AIT, using major natural components in the ELISA technique that allow better reaction than their modified or recombinant counterparts without the need of purified allergen components. For this reason, the measurement of specific serum IgG subclasses, particularly IgG4, should be considered as a good marker of protective or blocking antibody that may be useful for monitoring activation of tolerance-inducing mechanisms in patients under AIT.

Therefore, according to the results described above, reverse ELISA has shown to be a sensitive and alternative method for measuring natural allergen-specific serum IgG antibody subclasses, especially IgG4, providing valuable information for monitoring patients with allergic respiratory disease during AIT with peptides or native or recombinant allergens of clinical relevance.

6. Conclusion

We can conclude that IgE-mediated allergic patients submitted to AIT usually demonstrate immunological changes, in particular, induction of allergen-specific IgG that may act as blocking factors competing with IgE antibodies and thus contributing for ameliorating the clinical symptoms. In this context, we recommend follow these patients under AIT using clinical (symptoms and medication scores) and laboratorial (allergen-specific IgG subclass measurement) parameters since this technique has shown to be a potential tool for monitoring these patients.

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Author details

Ernesto Akio Taketomi*, Juliana Silva Miranda, Jair Pereira da Cunha-Júnior and Deise Aparecida de Oliveira Silva

*Address all correspondence to: etaketomi@gmail.com

Laboratory of Allergy and Clinical Immunology, Department of Immunology, Institute of Biomedical Sciences, Federal University of Uberlandia, Uberlandia, Brazil

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